RESEARCH ARTICLE

Immunomodulatory Effects of Probiotic *Bacillus siamensis* (ZMT02) on the Gross, Histomorphology and Histometric Indices of the Bursa of Fabricius in Broiler Chicken

Yamini Sundaramoorthy[1], Senthamil Selvan Paramadayalan[1]*, Rajalakshmi Kaliyaperumal[1], Nithya Quintoil Mohanadasse[2], Varun Arasan[3], Susim Mukul Ray[4]

Abstract

Bursa of Fabricius is the primary lymphoid organ and is the source of B lymphocytes for humoral immunity against pathogens in birds. Probiotic feeding as an alternate to antibiotic use is gaining impetus. The present study reports the immunomodulatory effects of *Bacillus siamensis* (ZMT02) as a potential probiotic supplement Vs. Zinc bacitracin on the gross morphology, histomorphology and morphometric indices of the Bursa of Fabricius in broiler chicken that were challenged with pathogenic *E. coli* and *Clostridium perfringens*. At slaughter (42 days post feeding), the gross and histomorphological features of the bursa of Fabricius in control, antibiotic (Zinc bacitracin 500 mg/ton feed feed) supplemented and probiotic (*Bacillus siamensis* ZMT02, 1000 mg/ton) supplemented groups were normal without any abnormalities. Morphometric and volumetric measurements were comparatively larger and heavier in the control group and were smaller and lowest for the antibiotic group. There was no significant difference in the number of follicles between groups but the average follicular diameter was highest for the probiotic group with a distinct and well developed thicker cortex and was lowest for the antibiotic supplemented group with a thinner and poor cortex. Antibiotic supplementation had affected the proliferation and maturation of the B cells that resulted in smaller follicles with lean cortex. Probiotc supplementation with *Bacillus siamensis* was immunostimulatory and induced formation of larger follicles with a well developed cortex. The higher weight and volume of the bursa at slaughter in control birds might have resulted from the higher capillary network and repopulated immune cells after a transient immunodepletion.

Key words: *Bacillus siamensis*, B lymphocytes, Bursa of fabricius, Immunomodulation, Zinc bacitracin

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Introduction

The use of antibiotic as prophylaxis in poultry production is in decline (Al-Khalaifah, 2018; Hidayat *et al.*, 2020) and beneficial micro-organisms are now being identified for their use as supplements to improve the gut microbiota in chickens. These non-pathogenic microorganisms are usually normal inhabitants of the intestinal flora and when administered at optimum levels, promote health by inhibiting the pathogenic microorganisms (Qamar *et al.*, 2020). These organisms include bacteria, fungi, yeast and bacteriophages. These probiotics have a positive effect on growth, immunity, gut microbiota and gut integrity. In this series, *Bacillus siamensis* is a newly identified and characterized bacterium. *Bacillus siamensis* LF4 improves immunity in fishes by enhancing anti-inflammatory cytokines, antimicrobial peptides, and downregulating the expression of pro-inflammatory cytokines (Liu *et al.*, 2024[a]). *Bacillus siamensis* Strain B28 has been recommended as probiotic in humans for its antimicrobial activity against foodborne pathogens (Heo *et al.*, 2021). The FVP1 strain of *Bacillus siamensis* improved fermentation and nutritive value of soybean products and has antibacterial effect against *Escherichia coli* and *Staphylococcus aureus* (Homsuvan *et al.*, 2023). In piglets it is reported that it reduced the pathogenic bacteria in the gut and has improved the gut microbiota and immunity (Liu *et al.*, 2024[b]).

[1]Department of Veterinary Anatomy & Histology, Rajiv Gandhi Institute of Veterinary Education & Research (RIVER), Kurumbapet, Puducherry-605 008, India

[2]Department of Veterinary Public health & Epidemiology, RIVER, Kurumbapet, Puducherry-605 008, India

[3]Department of Livestock Production and Management, RIVER, Kurumbapet, Puducherry-605 008, India

[4]Technical and Promotion (PBU), Zenex Animal Health India Private Limited, Ahmedabad, India

*__Corresponding author__: Senthamil Selvan Paramadayalan, Department of Veterinary Anatomy & Histology, Rajiv Gandhi Institute of Veterinary Education & Research (RIVER), Kurumbapet, Puducherry-605 008, India, E-mail: drsenvet@gmail.com

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Bursa of Fabricius is the central lymphoid organ in chickens for the proliferation and development of B lymphocytes. The humoral immunity in birds depends on the development

and normal functioning of this lymphoid organ. The follicular development in the bursa of Fabricius depends on the transport of active antigen and bacterial mitogen through the bursal lumen and stimulation of B cells in the lymphoid follicles (Ratcliffe, 2006). Studies on the beneficial effects of this probiotic organisms (*Bacillus Siamensis* ZMT02) in commercial broiler production as a replacement for antibiotic are not yet done and there is no study yet that details on the immunostimulatory effects of this organisms on the bursa of Fabricius. This study was aimed to report in detail on the gross morphological and micromorphological effects in the bursa of Fabricius of broilers supplemented with this probiotic and to compare with the antibiotic supplemented and control groups in Indian conditions.

Materials and Methods

Materials and Treatment

This experimental study was conducted following animal ethics and welfare and was approved by the Institutional Animal Ethics Committee, RIVER, Puducherry (India). The broiler chicks for the current study were procured from a commercial hatchery in Tamil Nadu, India. A total of 30 apparently healthy broiler chicks were selected and used. The chicks were randomly divided in to three groups (one control G1 and two experimental groups, G2, G3) and were housed and reared under good farming practices in deep litter system for a period of 42 days in the experimental sheds at the Livestock Farm Complex, RIVER, Puducherry. The chicks in control group G1 were fed basal diet, whereas those in treatment group G2 received basal diet + Zinc bacitracin @ 500 mg/ton feed and group G3 were fed basal diet + Probiotic @ 1000 g/ton of feed for the entire rearing period until slaughter.

The probiotic contained *Bacillus siamensis* (ZMT02, 2000 billion spores/kg). All the groups were challenged with pathogenic *E. coli* (MTCC No. 1610 @ $2.0x10^8$) on 7^{th} day and *Clostridium perfringens* (MTCC No. 450 @ $1.0x\ 10^8$) on 19^{th}, 20^{th} and 21^{st} day. The probiotic and the pathogenic strains of bacteria were supplied by Zenex Animal Health India Private Limited, Ahmedabad, Gujarat.

Gross Morphology and Morphometry

The birds were sacrificed on 42^{nd} day of age and their bursa of Fabricius was collected for gross morphological and morphometric observations. The collected bursa was rinsed in normal saline and carefully trimmed to remove for the adhering fat and connective tissue on its surface. Morphometric measurements such as the length, width, weight and volume were recorded with the Vernier caliper, thread, scale and digital weighing machine. Volumetric measurements were done by water displacement technique. The bursa was transversely cut to record the number of plica.

Histology and Histometry

One half of the bursa was used for histological studies. They were fixed in 10% Neutral Buffered Formalin and were processed for routine paraffin sectioning for histological examinations. Paraffin sections of 4-5 µm thickness were made with a microtome and were stained for standard hematoxylin and eosin staining technique (Bancroft and Stevens, 1996). The histomorphologic details of bursa of Fabricius in these three groups were studied and photomicrographed with Nikon H600L Photomicroscope (Japan) at CIF, RIVER. Histomorphometric measurements on the follicular density and size were done using a calibrated ocular micrometer.

Statistical Analysis

All the measurements recorded (Gross morphometry and micrometry) were tabulated with values of Mean and Standard Error and the statistical significance ($p<0.05$) between groups was analysed by ANOVA, with Tukey HSD test for multiple comparisons using SPSS software.

Results and Discussion

Gross Morphology and Morphometry

The gross appearance of the bursa in birds of all the three groups was seen as smooth, oval/globular whitish structure and was without any discoloration and associated pathologies. The size of bursa was comparatively larger in male than in the female birds of all the three groups. The central lumen was free and communicated with the proctodium of the cloaca. The length of the bursa measured more in the control, and was significantly different from the antibiotic and probiotic groups. Their width didn't differ between the groups. The weight and volume of the bursa was significantly higher of the control group than the other two groups. It was lowest for the antibiotic group (Table 1). This suggested that supplementation with zinc bacitracin had affected the gut microbiota and suppressed the stimulation of immune cells for follicular growth. Krinke and Zamroj (1996) also reported that feeding antibiotic avoparcine in broiler chickens caused hypocellularity in bursal follicles.

In present study probiotic supplementation with *Bacillus siamensis* from day one didn't affect bursal gross morphometric indices after pathogenic challenge with *E. coli* and *Clostridium* spp. In fact it was significantly lower than the control group (Table 1). The effect of probiotic on bursa varied in different studies. No significant difference in bursal weight was observed with probiotic supplementation with *Bacillus subtilis, Lactobacillus acidophilus,* and yeast *Saccharomyces cerevisiae* and prebiotic (Humic acid and Sodium humate) (Ahfeethah *et al*., 2023). Feeding a mixed probiotic of (*Lactobacillus paracasei* KL1 and *Lactobacillus plantarum* subsp. *plantarum Zhang-LL*) from day one of hatch in chicken significantly affected the bursal indices but, in

chicks that were fed later, *i.e.,* only after pathogenic challenge didn't affect the bursal indices (Chen *et al*., 2020).

Table 1: Gross morphometric measurements of bursa of Fabricius in control, antibiotic and probiotic groups (n=6)

Parameters	Negative control	Antibiotic	Probiotic
Weight (gm)	2.86±0.03^{ab}	1.32±0.08^{ab}	1.91±0.38^{ac}
Length (mm)	2.96±0.08^{ab}	2.13±0.18^{ab}	2.28±0.22^{ac}
Width (mm)	2.16±0.08	1.74±0.13	1.83±0.15
Volume (cc)	2.88±0.11^{ab}	1.21±0.12^{ab}	1.71±0.36^{ac}

Mean ±SE values bearing different superscripts within the row differ significantly (p<0.05).

Histomorphology and Histometry

Histological examination of the bursa of Fabricius in all the groups showed that the bursa possessed all the four histological tunics, *viz*., tunica mucosa, tunica submucosa, tunica muscularis and tunica serosa (Fig. 1). The lumen of the bursa was clear and was without any dead and inflammatory cells. The tunica mucosa showed longitudinal folds and was lined by columnar (follicular) epithelium and pseudostratified columnar (interfollicular) epithelium as reported by Banks *et al.* (1993). This follicular epithelium above the bursal follicles helps to present the antigen to the medullary B lymphocytes present inside the bursal follicles (Eurell and Frappier, 2013). Romanovych *et al.* (2019) found that the surface epithelial cells in control group were necrotic, desquamated and were found in the lumen of the bursa. This resulted in formation of microcystic cavities in the surface epithelium. However, in our study the follicular epithelium with its supportive cells and the cortico-medullary epithelial cells were intact and suggested that they supported antigen presentation in all the three groups (Fig. 2, 3).

The mucosal folds/ plica were extended in to the lumen of bursa of Fabricius. The height of this plica varied. There seemed no specific pattern in the arrangement of this tall and short plica between groups. The average number of plica was 17.33± 0.76, 22.7± 2.18 and 19.33± 0.84 in the control, antibiotic and probiotic groups, respectively (Table 2), which was in agreement with the Schat *et al.* (2014). At the center of each plica were the axial connective tissue septa that supported the blood vessels, nerves and lymphatics. From this septa extended the interfollicular septa that were seen between the bursa follicles on each side of the axial septa (Fig. 1).

Each plica was filled with bursal follicle of different shapes and sizes. The number of follicles varied between plica, individuals and groups. The average number of follicle in the tallest plica in control, antibiotic and probiotic group was 57.67±1.57, 59.83±7.16 and 41.22±6.42, respectively (Table 2) and was not statistically different between groups. The follicle in all groups showed an outer darkly stained cortex predominated by mature B lymphocytes and an inner medulla composed of reticular cells, immature and differentiating lymphoblast and stained paler. Between the cortex and medulla was a dense capillary network formed in the cortico-medullary border (Fig. 2). This capillary network was extensive in the control birds which could have resulted in its higher weight and volume. The cortico-medullary epithelium separated the outer cortex from the inner medulla. They are responsible for the formation of blood bursal barrier (Schat *et al*., 2014). The diameter of the follicles varied within a plica and individuals of all the three groups. In our study the average diameter of the follicles in control, antibiotic and probiotic treated groups was 416.76±18.19, 289.16±22.39, 425.37±30.75 µm, respectively (Table 2).

Table 2: Difference in follicle number and follicular diameter in control, antibiotic and probiotic groups

Bursal follicle	Negative control	Antibiotic	Probiotic
No. of plica	17.33±0.76	22.17±2.18	19.33±0.84
No. of follicle	57.67±1.57	59.83±7.16	41.22± 6.42
Follicular diameter (µm)	416.76±18.19^{ab}	289.16±22.39^{abc}	425.37±30.75^{bc}

Mean ±SE values bearing different superscripts within the row differ significantly (p<0.05).

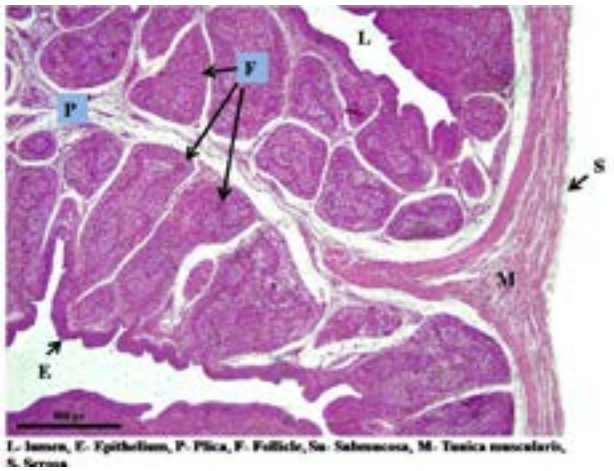
The follicular diameter was highest and significantly differed in the probiotic treated group (Table 2). Within the follicle the cortical areas were well developed and differentiated in the probiotic group and were suggestive of the proliferative effects of the probiotic on the bursal follicles in this group. This agreed with the findings of Romanovych *et al.* (2019), with probiotic *Saccharomyces cerevisiae* and Shah *et al.* (2018) with a combination of zinc and mixed probiotic protexin resulted in optimal bursal morphology with more follicles and cellular density. But, Rehman *et al.* (2020) reported that mixed probiotic (*L. plantarum, Lactobacillus rhamnosus, Enterococcus faecium, Candida pintolepesii, Bifidobacterium bifidum and A. oryzae*) feeding had no significant effect on the performance, internal organs and blood parameters of the chickens challenged with *Clostridium perfringens*. This difference indicates that multiple intrinsic and extrinsic factors are involved in evoking a successful humoral immune response.

The diameter of the follicle in the antibiotic treated group was significantly lowest. Within the follicles the cortex was thin and poorly differentiated (Fig. 3). This could be due to the negative impact of the antibiotic on the gut microbiota and its failure to induce the B cells in the medullary region to proliferate and mature for their migration in to the cortex. Only differentiated B cells that express a complete B-cell receptor can cross the cortico-medullary border to reach the cortex. B cells that lose the expression of these receptors die by apoptosis (Paramithiotis and Ratcliffe, 1994). Similar finding was reported by Chrząstek *et al.* (2011), who found that antibiotic supplementation in the first week of chick affected the immune response. This treatment especially decreased the number, percentage and distribution of B cells in the bursa of fabricius (Jankowski *et al*., 2022).

Nakamura *et al.* (1986) found that in birds challenged with *E. coli* there were lymphocytic depletion from the bursal follicles from the medullary and cortical regions. This effect was transient and repopulation of the follicular cells occurred and the bursa attained their original weight on day 14 post-challenge. In our study these repopulated cells could have compensated for the lymphoid depletion and therefore improved the bursal weight in the control birds after pathogenic challenge with *E. coli* and *Clostridium* spp. In another study, Romanovych *et al.* (2019) found that there were marked necrosis of the follicles and the central part of some of the lymphoid follicle possessed necrotic detritus in control birds. No such pathologies / pathogenic microorganisms were detected in our study (Fig. 4).

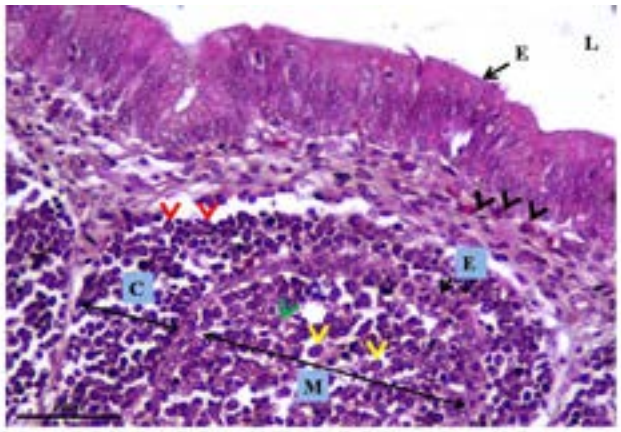
Conclusion

The results from our study showed that probiotic feeding with *Bacillus siamensis* (ZMT02) positively affected the gut microbiota and caused immunoproliferation to produce follicles of significantly higher diameter than the other two groups. Antibiotic supplementation with zinc bacitracin resulted in smaller bursa with lowest weight and volume than the other two groups, and negatively affected the microbiota induced B cell proliferation, number, maturation and their migration into the cortex. The higher bursal volume and weight in the control group was not accompanied with a significant increase in the number of bursal follicles and follicular diameter. We conclude that *Bacillus siamensis* can be a potential probiotic in the near future given either as a single or a mixed probiotic in the broiler ration. Further studies to identify the possible mechanisms of its beneficial effects as a probiotic organism are recommended.



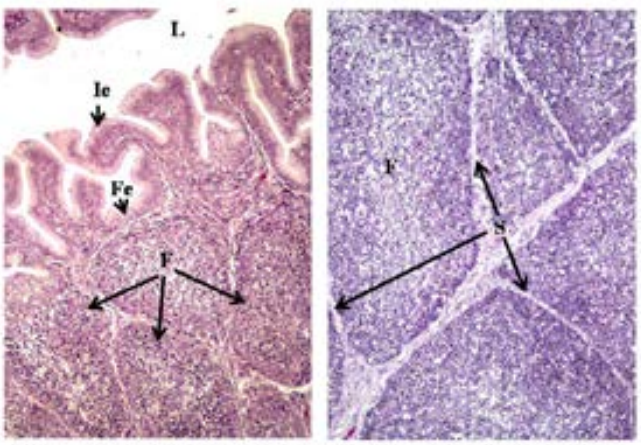
L- lumen, E- Epithelium, P- Plica, F- Follicle, Su- Submucosa, M- Tunica muscularis, S- Serosa

Fig. 1: Photomicrograph of the bursa of Fabricius in control group showing its histological layers (H & E- 4X)



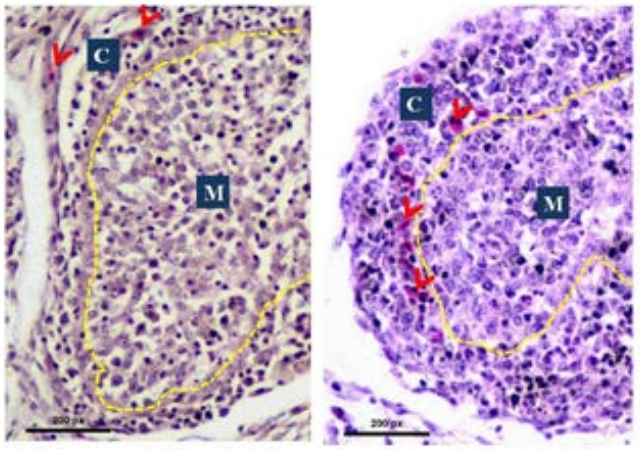
L- lumen, E- Epithelium, C- Cortex, M- Medulla, E- Coticomedullary epithelium, arrow heads- black- RBC's, Red- mature B cells, Yellow- lymphoblasts, Green – dendritic cells.

Fig. 2: Photomicrograph of the follicle in bursa of Fabricius of control group showing its histological features (H & E- 40X)



L- Lumen, Ie- Interfollicular Epithelium, F- Follicle, Fe- Follicle associated epithelium, S- Interfollicular Septa

Fig. 3: Photomicrograph showing difference in follicular size and number in bursa of Fabricius of Antibiotic and Probiotic group (H & E- 40X)



C- Cortex, M- Medulla, Yellow line - Coticomedullary epithelium, arrow heads- red- RBC's,

Fig. 4: oto i ro r o t e urs o i es o in i eren e in e u r isto o i ones in Anti ioti n ro ioti rou - 0

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